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Diversity Analysis in Indian Cooking Bananas (*Musa*, ABB) through Morphotaxonomic and Molecular Characterisation

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Abstract

Improvement of ABB bananas is one of the breeding objectives of the National Research Centre for Banana (NRCB), India. India is one of the main centres of origin and domestication of *Musa balbisiana*. The ability of the species to introgress naturally with *Musa acuminata* has contributed to the wide diversity of B and B-rich genomes (like ABB) in India. The NRCB field genebank has a vast collection of 125 cooking banana accessions, dominated by the Monthan and Bluggoe subgroups. Phylogenetic studies offer the potential for unveiling the diversity available in the cooking bananas for exploitation in genetic improvement programmes. In the present study, the ABB germplasm was characterised through morphotaxonomic traits and molecular markers, with AA and BB genomes included as reference groups. The data were subjected to Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA) using NTSYS (Rohlf, 1998). Out of 125 accessions characterised morphotaxonomically, 71 accessions were distinct, whilst the rest (54) were synonyms. Forty-eight accessions, suspected to be synonyms based on morphotaxonomic characterisation, were characterised using 36 pairs of microsatellite markers. In both systems, cluster analysis resulted in two major clusters. Cluster 1 comprised AA accessions and cluster 2 included BB and ABB accessions. Though Monthan and Bluggoe are the major subgroups, the current study also resulted in another subgroup called Bontha, which clustered along with Monthan at 40% similarity. The estimated Cophenetic correlation coefficients were significant (0.98) in both molecular and morphotaxonomic characterisation, indicating a good fit of the dendrogram with the similarity matrices produced. The two-dimensional scatter plot obtained as a result of PCA also confirmed the clustering patterns elucidated by HCA. The phylogenetic relationships derived in the present study complemented with fertility studies facilitate identification of diverse parents, useful for the development of superior hybrids. These data also facilitated the elimination of synonyms during the establishment of a comprehensive core collection at NRCB.

INTRODUCTION

Annual production of bananas in the world is around 125 million tonnes (FAO, 2010). Around 43% are dessert bananas and 57% are cooking bananas (Uma et al., 2005). In India, the cultivation of ABB cooking bananas is restricted to local consumption. Nine subgroups have been identified, but the majority of the ABB cooking bananas belong to one of five subgroups: Bluggoe, Monthan, Peyan, Pisang Awak and Neymannan (Uma et al., 2005). They possess desirable traits, like resistance to biotic and abiotic stresses.

India, with its vast and varied *Musa* diversity, has been entrusted the responsibility of improving ABB cooking types by the global *Musa* breeding community. This requires basic information on diversity and phylogenetic relationships which are considered valuable for the identification of cultivars, detection of the genetic structure of germplasm, identification of diverse parental combinations to develop segregating progenies with maximum genetic variability for further selection, etc.

Valsalakumari et al. (1984) conducted variability and genetic divergence studies among Indian bananas, while Swennen et al. (1995) studied the pattern of variability among African plantains. Later, morphotaxonomic characterisation was addressed by Menon and Premalatha (2000). The results of the above studies revealed the relationships between individuals, and enabled the identification and elimination of synonyms. Uma et al. (2005) morphotaxonomically characterised the 545 germplasm accessions in the *Musa* collection of the National Research Centre for Banana (NRCB) for 121 traits using the descriptors for banana (IPGRI-INIBAP/CIRAD, 1996). Morphological traits are, however, often influenced by genotype x environment (GxE) interactions and the stage of plant growth, limiting their use in taxonomy (Ortiz, 1995). Moreover, morphological characters may also be controlled by only a small number of genes that do not represent the total genetic diversity within the genome (Brown-Guedira et al., 2000). Hence, the current focus is on molecular markers which are free from GxE interactions and ensure authentic identification of germplasm accessions. In addition, genetic differences can be detected even during early stages of development, an advantage over most phenotypic markers that do not allow revealing the differences until the crop has attained a certain stage of growth.

Microsatellite markers have been utilised for numerous applications in *Musa* to facilitate and enhance the handling and improvement of germplasm. They have proven especially useful for germplasm characterisation (Creste et al., 2004; Noyer et al., 2005), marker-assisted selection and linkage map construction (Ciampi et al., 2004). Attempts were therefore made in the present study to analyse the diversity and phylogenetic relationships in ABB germplasm through morphomolecular characterisation, potentially applicable to the strategic improvement of *Musa*, a crop which is highly recalcitrant for breeding due to inherent problems such as parthenocarpy, sterility and polyploidy.

MATERIALS AND METHODS

Morphotaxonomic data for 131 traits of 148 germplasm accessions, including 126 ABB, 4 AA and 18 BB accessions, were subjected to multivariate Hierarchical Cluster Analysis (HCA) and Principal Component analysis (PCA) using NTSYS Version 2.01e (Rohlf, 1998).

Fresh cigar leaves were collected from the field genebank of NRCB, Trichy. Genomic DNA was isolated using hexadecyltrimethyl ammoniumbromide (CTAB) as described by Gawal and Jarret (1991) with minor modifications. Following purification, DNA was quantified using a Perkin Elmer spectrophotometer (UV-VIS Lambda 25, USA). Polymorphism in ABB germplasm was analysed using 36 tested microsatellite primer pairs.

The PCR protocol of Lagoda et al. (1998) was adopted with minor modifications. Both normal and Touch Down PCR's (TD-PCR) were used depending on the availability of primer information. PCR reactions were performed on an Eppendorf gradient master cycler (Eppendorf, Hamburg, Germany) in 25-µl reactions containing 50 ng of genomic DNA 100 µM of each of the dNTPs, 0.2 µM of each primer, 1X PCR buffer (100 mM

Tris-HCl, pH 9.0, 500 mM KCl, 10 mM $MgCl_2$, 0.1% gelatin) and 1.5 U Taq DNA polymerase.

Allele separations were performed on a 38 x 30-cm sequencing unit (Sequi-Gen-GI, Bio-Rad, Australia,) using Urea-PAGE (5%). Electrophoresis was performed at 2300 V, 75 mA, 60 W and at VHR'S ranging from 2000 to 3500 depending on the product size. The gels were silver stained as described by Creste et al. (2004).

PCR-amplified products from individual accessions were scored as a dominant marker for the presence (1) or absence (0) of an allele. The Genetic Similarity (GS) matrix was examined by hierarchical cluster analysis (HCA) using the Unweighted Pair Grouping Method with Arithmetic averages (UPGMA) and Sequential Agglomerative Hierarchical and Nested (SAHN) clustering methods and plotted in a phenogram using NTSYS Version 2.01e. The cophenetic correlation (R value) coefficient was used to test for association between the clusters in the dendrogram and the similarity matrix. The Mantel test of significance was used to compare the similarity matrices produced by the morphotaxonomic and molecular data.

RESULTS AND DISCUSSION

In both morphotaxonomic and molecular characterisation, cluster analysis resulted in two major clusters. Cluster 1 comprised AA accessions and cluster 2 included BB and ABB accessions. Morphotaxonomic characterisation of 148 accessions resulted in the identification of 69 distinct accessions, while the rest were synonyms (Fig. 1). This is in agreement with the reports of Menon and Premlatha (2000) who were able to identify only 96 distinct clones out of 207 accessions characterised using the descriptors for banana. The synonyms identified could be eliminated during the establishment of a core collection after retaining one true representative for each of the synonymous groups.

Microsatellite Diversity

Among the 36 primer pairs tested, 30 pairs (83.33%) that produced discrete, repeatable amplicons were considered for genetic diversity analysis. A total of 158 alleles were identified among the tested accessions with a mean of 5.27 alleles per primer pair. The size of the fingerprints ranged from 100 to 600 bp. The observed number of alleles was more than expected in both the diploid and triploid accessions for three microsatellite markers. Similar results have been reported by Crouch et al. (1999), who suggested that it was mainly due to the high frequency occurrence of duplicated alleles or chromosomal regions in A and B genomes of *Musa*. Similarly, the observed and expected product range were not always in accord, namely with primer pairs AGMI 133/134, Mb SSR 1-69, Mb SSR 1-113, Mb SSR1-49-2, Mb SSR 1-12, Mb SSR 1-50 and Mb SSR 1-156. The possible reasons for such discrepancies might be the denaturation conditions during electrophoresis, the type of molecular weight marker used as standard or differences in the base pair composition of the microsatellite primers, as suggested by Testolin et al. (2000).

Polymorphic Information Content (PIC) values ranged from 0.3011 to 0.5733 with an average of 0.41. PIC above 0.5 was registered in 30% of the primer pairs. These values were comparatively higher for the AGMI series of primers used. Higher PIC values could be attributed to the high level of polymorphism. This is in conformity with earlier reports of Struss and Plieske (1998).

Forty percent of the primers failed to amplify products in some of the test accessions. The failure of product amplification has been a common phenomenon in

Musa (Crouch, 1999). Production of null alleles or total restriction of the amplification could be attributed to the following reasons: (1) variations in the sequences flanking the microsatellite markers as reported by Grapin et al. (1998), and (2) distant phylogenetic relationship between cultivars and wild accessions used as source for microsatellites (Carreel et al., 2002).

Alleles specific for genomes AA (*Musa acuminata* ssp. *burmannicoides* 'Calcutta 4'), BB (*Musa* balbisiana 'Elavazhai') and ABB cultivars ('Sagarkol', 'Pidi Monthan', 'Boothibale', 'Bluggoe' and 'Nuchan-I') have been identified with the microsatellite primer pairs used for genetic diversity analysis (Fig. 2). Such primer pairs could be used as markers to confirm the individual's identity in the context of Intellectual Property Rights (IPR) issues. Unique alleles could be converted into genetic probes, and used in the selection and identification of *Musa* germplasm accessions (Wong et al., 2001). Few alleles were commonly shared by AA and ABB while some were common to BB and ABB. Results suggest the involvement of 'Calcutta 4' (0642) and 'Elavazhai' (0167) in the evolution of ABB test accessions and established the genetic relationships between triploid and diploids as reported by Creste et al. (2004). PCA revealed a similar clustering as obtained in the dendrogram based on cluster analysis as reported by Karamura (1999) in her numerical taxonomic studies of the East African highland bananas in Uganda.

The cophenetic correlation coefficient estimated was 0.98 both for molecular and morphotaxonomic characterisation, indicating a good fit of the dendrogram with the similarity matrices produced. Mantel T-test of significance, used to compare the two matrices, produced a linear model indicating that the results of morphotaxonomic characterisation are congruent with those of molecular characterisation.

Though genome-specific microsatellite markers were used in the present study, they amplified products in all AA, BB and ABB accessions irrespective of their specificity. *Musa acuminata*-specific microsatellite markers, however, were less polymorphic in *M. balbisiana* and vice-versa, as reported by Sotto and Volkaert (2004).

The banding profile observed in the present study did not permit to define the allelic relationships among germplasm accessions, due to errors made in scoring microsatellite polymorphism in a polyploid and heterozygous species like banana in which each allele is interpreted as a unique character without considering the gene dosage (Provan et al., 1996).

Cluster Analysis

Molecular characterisation using microsatellite markers showed two major distinct clusters (Fig. 3). Cluster 1, which included *M. acuminata* ssp. *burmannicoides*, stood distinctly away from other accessions sharing more than 40% dissimilarities. These results are in conformity with the clustering pattern based on morphotaxonomic characterisation. Though *M. acuminata* ssp. *burmannicoides* had its origin in the Indo-Myanmar region, especially in the Chittagong forest in Myanmar and Tripura, the extent of dissimilarity of *M. acuminata* ssp. with other accessions has raised doubts about its probable involvement in the origin of B-rich genomes of India. Use of advanced techniques like Fluorescence In Situ Hybridisation (FISH) might be able to resolve such uncertainty.

The second cluster encompassed most of the ABB clones. The reference clone 'Elavazhai' exhibited more than 30% similarity with the ABB accessions. 'Elavazhai' (0167) is a wild BB of Indian origin and had been in human intervention for several decades. The chances of its involvement in some stage of the evolutionary pathway of

cooking banana cannot be ruled out. In morphotaxonomic and molecular characterisation, the extent of similarity among the members of this cluster was 80% and 70% respectively, suggesting that the results are analogous.

Subcluster 2a was divided into four microclusters as against five in morphotaxonomic characterisation based on their extent of similarity and grouping pattern. Subcluster 2a had 26 accessions. The third and fourth microclusters comprised Bontha and Ashy Bontha types, sharing 40% similarities with the Monthan subgroup. The results suggest that Bontha types could be treated as a separate subgroup of cooking bananas.

'Monthan' (0293) and 'Kashkal' (0068) clustered with 88-90% similarities. The percentage similarities remained the same, even in morphotaxonomic characterisation d to bold, round-beaked fruits. The grouping of 'Battisa Local' (0088) with Monthan was expected, as 'Battisa Local' (0088) has almost similar traits to Monthan and differs only for the extended female phase.

Irrespective of their collection origin, 'Pidi Monthan' (0115), 'Karim Bontha' (0121), 'Chirapunji' (0163), 'Karibale' (0129), 'Bluggoe' (0158) and 'Monthan' (0349) grouped together in subcluster 2a with more than 96% similarities. Basically all are Monthan types with green, bold fruits and a rounded beak. It was not possible to establish a specific correlation between genetic similarities and geographical origin. This probably reflects the human intervention in the dispersion of *Musa* accessions (Creste et al., 2004). Although the three accessions mentioned above, i.e. 'Monthan' (0293), 'Kashkal' (0068) and 'Battisa Local' (0088), are similar to the aforesaid accessions, their deviation or dissimilarity by 7-8% is not clearly understood, but could be due to the score for one or two specific traits analysed. The authenticity of scoring and reliability on these specific traits need to be confirmed using more molecular markers.

'Kallu Monthan' (0171), 'Cherapadathi' (0239), 'Malai Monthan' (0402) and 'Madhok Grong' (0429) grouped together. Of these, 'Malai Monthan' and 'Madhok Grong' are Monthan types which have 96% similarities with nearby Monthan groups, consisting of 'Monthan' (0293), 'Kashkal' (0068), 'Battisa Local' (0088) and others. These also had 95% similarities with 'Kallu Monthan' (0171) and 'Cherapadathi' (0239), which are Bluggoe types. 'Kallu Monthan' (0171) and 'Cherapadathi' (0239) were 100% similar and hence termed as synonyms. 'Cherapadathi' is an ashy mutant of 'Kallu Monthan'. It is known that microsatellite markers cannot usually discriminate somatic mutants (Testolin et al., 2000). Results of molecular characterisation suggest that ashyness of fruits has not contributed to changes at the molecular level. Contrarily, morphotaxonomic characterisation has indicated more than 40% dissimilarities between green accessions and their ashy mutants. 'Kali Bale' (0170), 'Pacha Bontha Batheesa' (0189), 'Yenugu Bontha' (0221), 'Bluggoe' (0523), 'Ashy Batheesa' (0493), 'Dev Bale' (0363) and 'Erode Kai' (0415) had similarities ranging from 93 to 100%. 'Yenugu Bontha' is a Monthan type while 'Pacha Bontha Batheesa' is also a Monthan type but with an extended female phase. These morphological dissimilarities were not manifested at the molecular level and they were found to be synonyms. This corroborates results of Gawal et al. (1992) who reported that large variations in morphological characteristics need not necessarily reflect the same degree of genetic variation.

'Kostha Bontha' (0530), 'Chetty' (0553), 'Kallu Monthan' (0557) and 'Pacha Bontha Batheesa' (0222) are also Monthan types of which only 'Pacha Bontha Batheesa' (0578) is a Monthan type with extended female phase. 'Dakshin Sagar' (0289) clustered slightly away from other Monthan types of 2a with 12-14% dissimilarities.

Phenotypically and morphotaxonomically' little difference was observed. But molecular characterisation exhibited variation at genotypic level, which could be attributed to their high yielding abilities.

Surprisingly, 'Monthan' (0534) of Kerala origin exhibited more than 22% dissimilarities with all the Monthan types discussed above. Though, morphologically, it has traits similar to Monthan, the reason for its separate clustering might be due to unexpressed genotypic variability. But this result needs to be confirmed with more primers.

In subcluster 2b, 'Kait Long' (0060), 'Pagar Banana' (0102) and 'Birbutia' (0086) clustered along with 'Gauria' (0271), 'Kallu Monthan' (0366) and 'Sakkai' (0416) with more than 85% similarities. 'Kait Long' and 'Pagar Banana' expressed almost 90% similarities through morphotaxonomic characterisation. Similarly, 'Jatikal' (0751) and 'Sagarkol' (1010) exhibited 100% similarities under morphotaxonomic characterisation whereas molecular characterisation exhibited a genotypic variation of 40-45%. The results suggest that many of the genotypic traits are not expressed. Although 'Gauria' (0271), 'Kallu Monthan' (0366) and 'Sakkai' (0416) had grouped with more than 90% similarities, 'Kallu Monthan' is a Monthan type while 'Sakkai' and 'Gauria' are Bluggoe types. This grouping of Kallu Monthan separately away from other Monthan members needs clarification.

In subcluster 2c, 'Nepali Chinia' (0097), 'Kanchikela' (0172 and 0509), 'Bangrier' (0265) and 'Batheesa Ash' (0246) grouped with 82% similarities. The cluster comprised of 'Gauria' (0271), 'Kallu Monthan' (0366) and 'Sakkai' (0416) were separated from the Bangrier group including 'Kanchi Kela' (0172 and 0509) and 'Bangrier' (0265), and the extent of similarities was less than 10%. Even in the morphotaxonomic characterisation, the extent of similarity spread between the clusters remained the same. 'Batheesa Ash' (0246), a unique Monthan type, clustered with Bangrier types. Though morphologically it is an intermediate accession between Monthan and Bluggoe types, genotypically it seemed to be closer to the Bluggoe types. Morphotaxonomically, accessions 'Kanchikela' (0172 and 0509) and 'Bangrier' (0265) were found to be synonyms. This is in line with the findings of Baum (1981) who reported that it is not possible for the morphotaxonomic traits to detect the minor variations like those within the members of a subgroup. But molecular characterisation did not support the synonymity status of these accessions and the extent of dissimilarity was found to be more than 8%.

In subcluster 2d, 'Alukhel' (0398), 'Sambal Neyvannan' (0450), 'Veneetu Mannan' (0543) clustered together with more than 85% similarities in molecular characterisation but the same exhibited 100% similarities through morphotaxonomic characterisation. Though their origin seems to be the same, long periods of domestication in various agroclimatic conditions could have contributed to these variations through minor mutations.

Though 'Bluggoe' (0333) and 'Boothibale' (0552), members of the Peyan subgroup, were found to be synonyms through morphotaxonomic characterisation, they exhibited only 10% similarities at genotypic level. This discrepancy does not confirm to the present observations and needs further genetic analysis. 'Pidi Monthan' (0412), a Monthan type, clustered with Peyan type. But it is not tallying with morphotaxonomic characterisation and morphological observations. This discrepancy also needs further genetic analysis.

'Nuchan-I' (0749) is a unique ABB member with more than 60% dissimilarities with other ABB culinary types. This is more of a dessert cultivar with less starch and higher total soluble solids (TSS) content (32°B). The results of morphotaxonomic and molecular characterisation were analogous and comparable except under few circumstances. A high degree of polymorphism observed within the genomic groups possibly reflects the high degree of variability as reported by Carreel et al. (2002). Degree of polymorphism however was substantially reduced within the subgroups. These results are congruent with the findings of Creste et al. (2004).

CONCLUSION

The phylogenetic relationships derived in the present study complemented with fertility studies helped in the identification of diverse parents which could be used for the development of superior hybrids. This also facilitated the elimination of synonyms during the establishment of a comprehensive core collection at NRCB.

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Figures

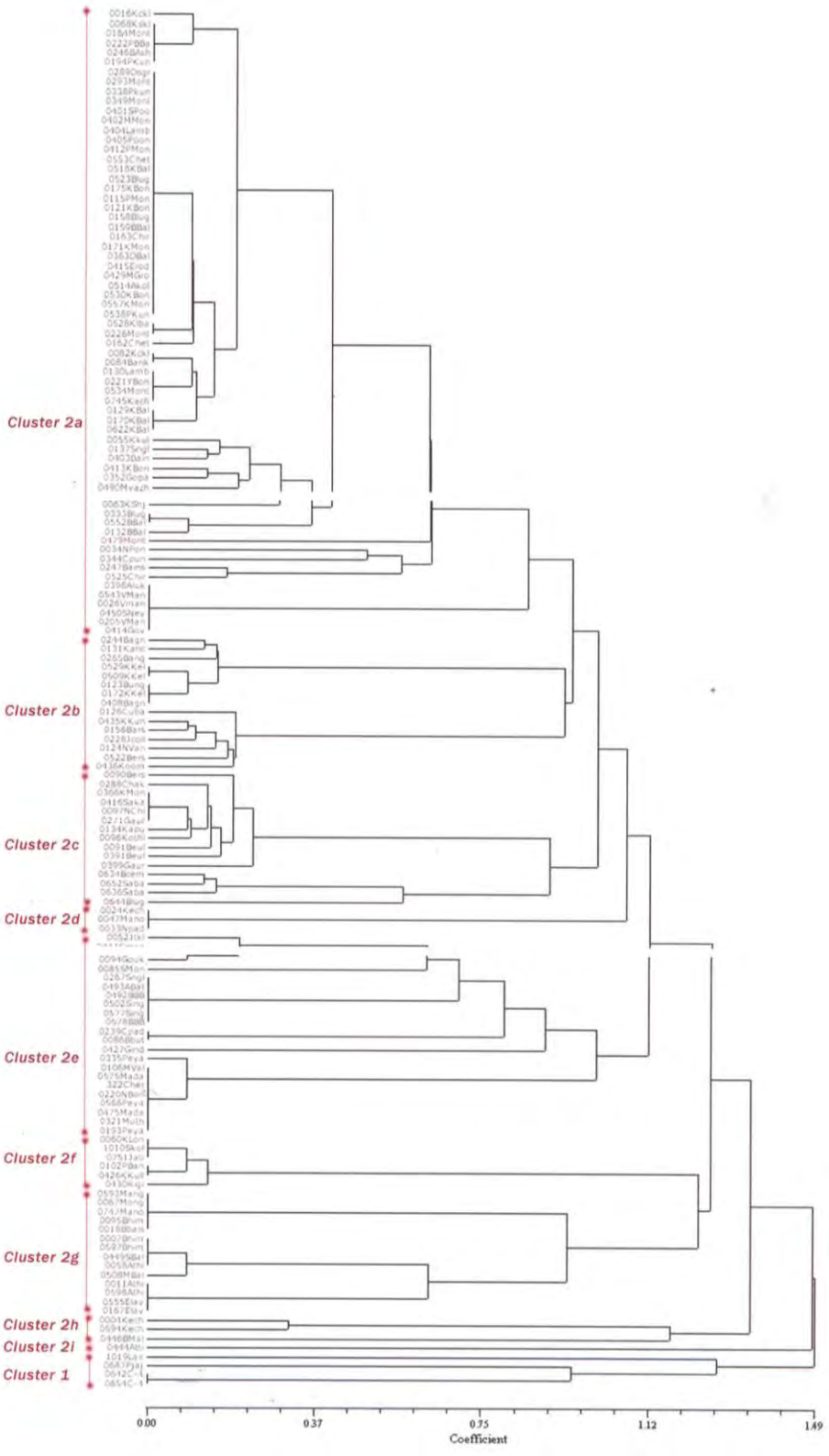

Fig. 1. Dendrogram resulting from cluster analysis of 148 *Musa* germplasm accessions based on 131 morphological traits.

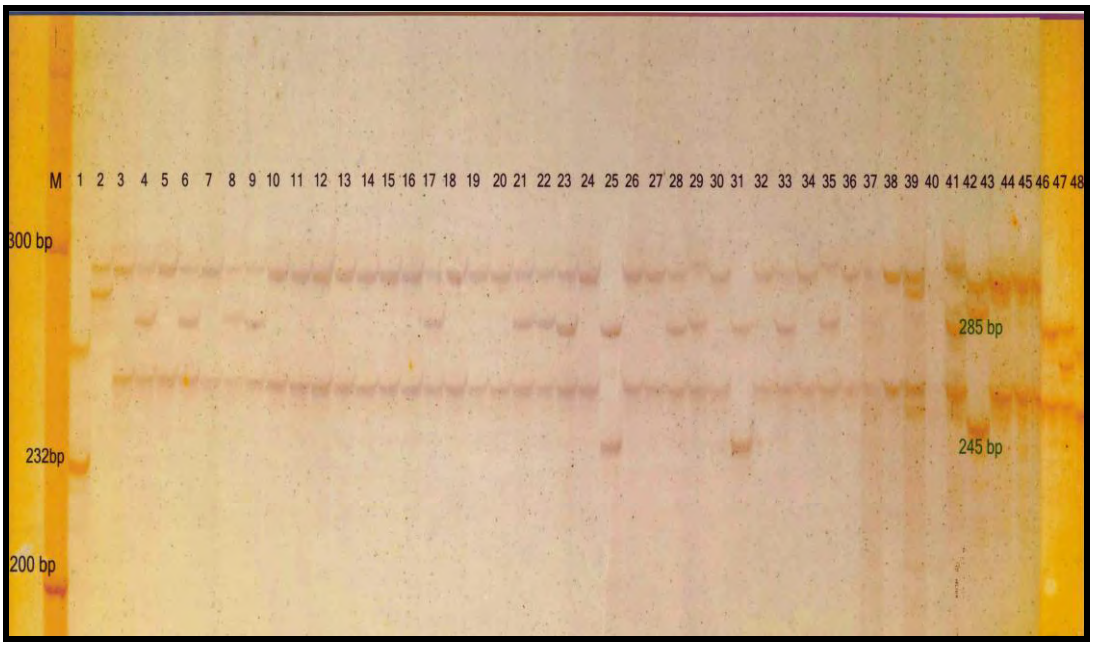

Fig. 2. DNA polymorphism for cooking bananas using microsatellite marker AGMI 24/25. M: Marker lane; 1-48: Test Accessions; Marker for A genome: 232 bp; Marker for Boothibale (0552): 245 & 285 bp.

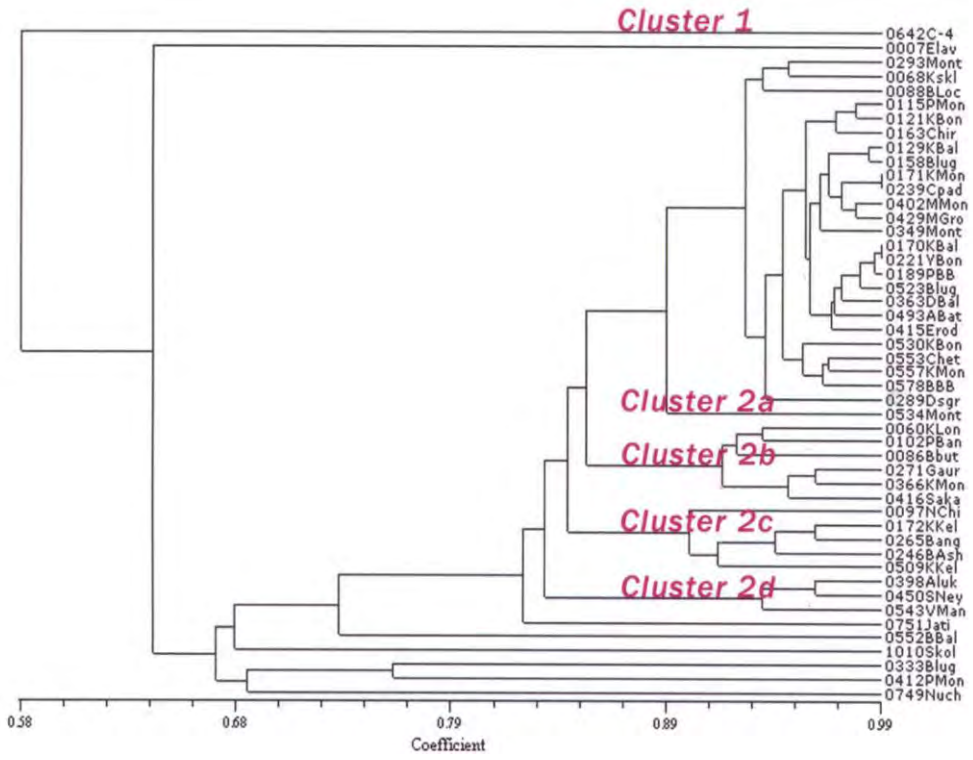

Fig. 3. Dendrogram showing genetic relationships among 48 *Musa* germplasm accessions based on Simple Matching coefficient (SM) obtained using 30 microsatellite markers.